FEBS Letters 584 (2010) 2473–2477

FEBS Letters

journal homepage: www.FEBSLetters.org

p53-dependent transcriptional regulation of EDA2R and its involvement in chemotherapy-induced hair loss

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ARTICLE INFO

Article history:
Received 21 March 2010
Revised 19 April 2010
Accepted 20 April 2010
Available online 29 April 2010

Edited by Angel Nebreda

Keywords:
XEDAR
Alopecia
Cyclophosphamide
Knockout
Chemotherapy-induced alopecia

ABSTRACT

The p53 tumor suppressor coordinates a multitude of cellular and organismal processes and exerts its activities mainly by activation of gene transcription. Here we describe the transcriptional activation of ectodysplasin A2 receptor (*EDA2R*) by p53 in a variety of cell types and tissues. We demonstrate that treatment of cancer cells with the ligand EDA-A2, known to specifically activate EDA2R, results in p53-dependent cell death. Moreover, we show that *EDA2R* is transactivated by p53 during chemotherapy-induced hair-loss, although its presence is not necessary for this process. These data shed new light on the role of *EDA2R* in exerting p53 function.
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1. Introduction

The transcription factor p53 is a pivotal tumor suppressor that integrates a wide variety of stress signalling pathways and is involved in numerous processes, including cell-cycle, apoptosis, differentiation, metabolism, senescence, etc. p53 exerts its activity primarily through transcriptional activation of target genes, few dozens of which were identified during the thirty years of p53 research [1]. Utilizing RNA interference to knockdown p53 in human embryonic fibroblasts, followed by global expression analysis, we identified ectodysplasin A2 receptor (*EDA2R*) as a potential transcriptional target of p53 in these cells.

EDA2R, also known as *XEDAR* (X-linked Ectodermal Dysplasia Receptor), encodes a transmembranal receptor that belongs to the tumor necrosis factor (TNF)-receptor superfamily [2]. EDA2R, as well as its paralog, EDAR, bind the ectodysplasin ligands EDA-A2 and EDA-A1, respectively; which are two alternatively spliced forms of the *EDA* gene [2]. Mutations in the *EDA* gene are associated with the X-linked form of Hypohidrotic Ectodermal Dysplasia (HED), a disease typically characterized by abnormal hair, teeth and sweat glands. Likewise, the autosomal form of HED may stem from mutations in the *EDAR* gene [3]. Although both *EDAR* and its ligand *EDA* are associated with HED, there are currently no reports of mutations in *EDA2R* in patients with this disease. Nevertheless, substantial data portray *EDA2R* as an apoptosis-inducing death receptor and implicate it in the regulation of ectodermal functions. For example, EDA2R was demonstrated to induce caspase-dependent apoptosis upon EDA-A2 treatment [4,5]. Moreover, in chickens, *EDA2R* is specifically expressed in placodes, the epithelial regions that give rise to feathers, and expression of a dominant-negative EDA2R suppresses feather development [6]; while in humans, a specific single nucleotide polymorphism in *EDA2R* coding region is associated with Androgenetic Alopecia (also known as male pattern baldness) [7]. In contrast to the reported roles of EDA2R in apoptosis, and its association with the growth of hair or feathers, knockout of *EDA2R* in mice was reported to have no phenotypic effect [8]. Interestingly, Tanikawa et al. had recently identified *EDA2R* as a p53 target gene that mediates anoikis, and suggested that *EDA2R* is a tumor suppressor that limits colorectal cancer progression [9].

Taken together, the aforementioned data led us to hypothesize that the transactivation of *EDA2R* might mediate p53-dependent

Abbreviations: CIA, chemotherapy-induced alopecia; HHFs, human hair follicles; MEFs, mouse embryonic fibroblasts; shRNA, small-hairpin RNA; WT, wild-type; KO, knockout; TNF, tumor necrosis factor

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doi:10.1016/j.febslet.2010.04.058

apoptosis upon EDA-A2 treatment. Moreover, the loss of hair following chemotherapy, a process known as chemotherapy-induced alopecia (CIA), was shown to be strictly dependent on p53 [10], and to involve apoptosis of hair follicles in response to the chemotherapy-induced DNA-damage [11]. Therefore, we speculated that *EDA2R* might mediate p53-dependent CIA. Hair-loss is one of the most common and traumatic adverse effects of chemotherapy [12], and currently it is virtually untreatable. Hence, studying the mechanisms that underlie CIA is of high importance, and may facilitate the development of novel therapeutic strategies.

2. Materials and methods

2.1. Cell culture

Immortalized WI-38 and IMR90 cells were cultured in MEM supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics. Mouse embryonic fibroblasts (MEFs) were derived from wild-type p53 (WT-p53) and p53 knockout (p53-KO) sibling embryos, and maintained in DMEM supplemented with 10% FCS and antibiotics. U2OS and H1299 cell lines were cultured in DMEM and RPMI, respectively, with 10% FCS and antibiotics.

2.2. Plasmids and infections

Small hairpin RNAs (shRNAs) targeting p53 (sh-p53), or mouse NOXA (sh-con) were stably expressed using pRetroSuper and were described in [13]. Mutant $p53^{R248Q}$ was generated by site-directed mutagenesis of pWZL-WT-p53 vector. Retroviral infection and the immortalization of WI-38 cells were described in [14].

2.3. EDA-A2 treatment and cell survival analysis

sh-p53 and sh-con U2OS cells were treated with EDA-A2 (R&D systems) at a concentration of 50 ng/mL for 24 h. Cells were stained with Crystal Violet and washed with PBS. Crystal Violet was extracted with acetic acid and quantified using a spectrophotometer at 590 nm. Cell death was estimated by Propidium Iodide (PI) exclusion: cells were trypsinized, stained with PI (50 ng/mL) and analyzed using a flow cytometer.

2.4. RNA preparation and QRT-PCR

RNA was extracted with TRI-Reagent (Molecular Research Center Inc.) and reverse transcribed using Bio-RT (Biolab) and random hexamers. Quantitative real-time PCR (QRT–PCR) was performed using Platinum SYBR Green qPCR SuperMix (Invitrogen). Human and mouse mRNA levels were normalized to the level of *GAPDH* and *HPRT*, respectively, of the corresponding sample. PCR reactions were performed in duplicates and error bars in charts represent the corresponding standard deviations. For analysis of mRNA expression in vivo, tissues from three mice were collected per experimental category, and error bars in charts represent S.E.M. (standard error of mean). Primer sequences are listed in Supplementary Table 1.

2.5. Western blotting

Western blot analysis was performed as described in Ref. [14] with the following antibodies: α-p53 pAb H-47, α-p21 sc-377 (Santa Cruz), and α-GAPDH MAB374 (Chemicon).

2.6. Mice and induction of alopecia in vivo

C57BL/6 p53-KO mice were kindly provided by Dr. G. Lozano (University of Texas, MD Anderson Cancer Center). EDA2R-KO mice were kindly provided by Genentech and were described by Newton et al. [8]. Induction of alopecia was performed as described in Ref. [15]. Briefly, back sections of 6–8 weeks old C57BL/6 anesthetized mice were depilated using 1:1 mixture of beeswax and gum rosin (Sigma). Nine days later, mice were injected i.p. with 150 mg/kg cyclophosphamide (CYP). Hair loss was monitored visually approximately one week after CYP injection.

3. Results

3.1. EDA2R is transcriptionally activated by p53 is human and mouse cells

As mentioned above, stable expression of a p53-specific shRNA (sh-p53) in WI-38 human embryonic fibroblasts led to a pronounced decrease in the expression of *EDA2R* compared to control shRNA-expressing cells (sh-con). To support the notion that *EDA2R* is transcriptionally activated by p53 in normal human cells, we treated sh-p53 and sh-con WI-38 cells with Nutlin-3a, a small-molecule that stabilizes p53 protein by inhibiting its Mdm2-dependent degradation [16], and measured the expression of *EDA2R*, as well as *p21* (*CDKN1A*) as a positive control for p53 activation. As depicted in Fig. 1A and B, in sh-con cells, Nutlin-3a enhanced p53 protein level and led to the transactivation of *EDA2R* and *p21*. This effect was completely abrogated in sh-p53 cells. The expression of *EDA2R* was further analyzed in the human fetal IMR90 primary lung fibroblasts. Indeed, p53 inactivation, either by shRNA or by expression of a dominant-negative DNA-contact mutant p53 (Fig. 1C), strongly attenuated the expression of *EDA2R* (Fig. 1D). p53 was capable of transcriptionally inducing *EDA2R* also in human cancer cells. Specifically, when the p53-null H1299 lung adenocarcinoma cells were transfected with either WT-p53 or the conformational mutant $p53^{R249S}$, a robust WT-p53-dependent induction of *EDA2R* transcription was observed. Similarly, treatment of WT-p53-expresssing HCT-116 colorectal carcinoma cells and their p53-KO counterparts with Doxorubicin, a widely-used chemotherapeutic agent, resulted in p53-dependent *EDA2R* induction (Supplementary Fig. 1). Furthermore, *EDA2R* was validated as a p53 target gene in additional cell lines, including U2OS osteosarcoma (Fig. 2A) and MCF7 breast adenocarcinoma (data not shown). Finally, MEFs, originating from either WT-p53 or p53-KO mice, displayed clear p53-dependent expression and Nutlin-3a-induced upregulation of *EDA2R* (Fig. 1E). In conclusion, EDA2R transcription is directly activated by p53 in a large variety of normal and cancerous cell types of human and mouse origin.

In agreement with Tanikawa et al. [9], we also observed that two adjacent p53 consensus binding sites in the first intron of *EDA2R* mediate its transcriptional activation by p53. As shown in Supplementary Fig. 2 and described in detail in the supplementary figure legend, chromatin-immunoprecipitation analysis revealed p53 binding to the region containing these two consensus binding sites. Luciferase reporter studies demonstrated that these sites are functional and only mutations in both sites could abrogate the p53-dependent activation of the reporter.

3.2. EDA-A2 treatment results in p53-dependent cell death

Chang et al. previously demonstrated that EDA2R can induce caspase-dependent apoptosis upon EDA-A2 treatment [5]. We therefore reasoned that p53-dependent induction of *EDA2R* may increase the sensitivity of cells to EDA-A2. To test this hypothesis we focused on WT-p53-expressing U2OS osteosarcoma cells and their sh-p53-expressing counterparts, which express lower levels of *EDA2R* (Fig. 2A). Treatment of these cells with recombinant EDA-A2 resulted in a p53-dependent and EDA-A2-induced cell

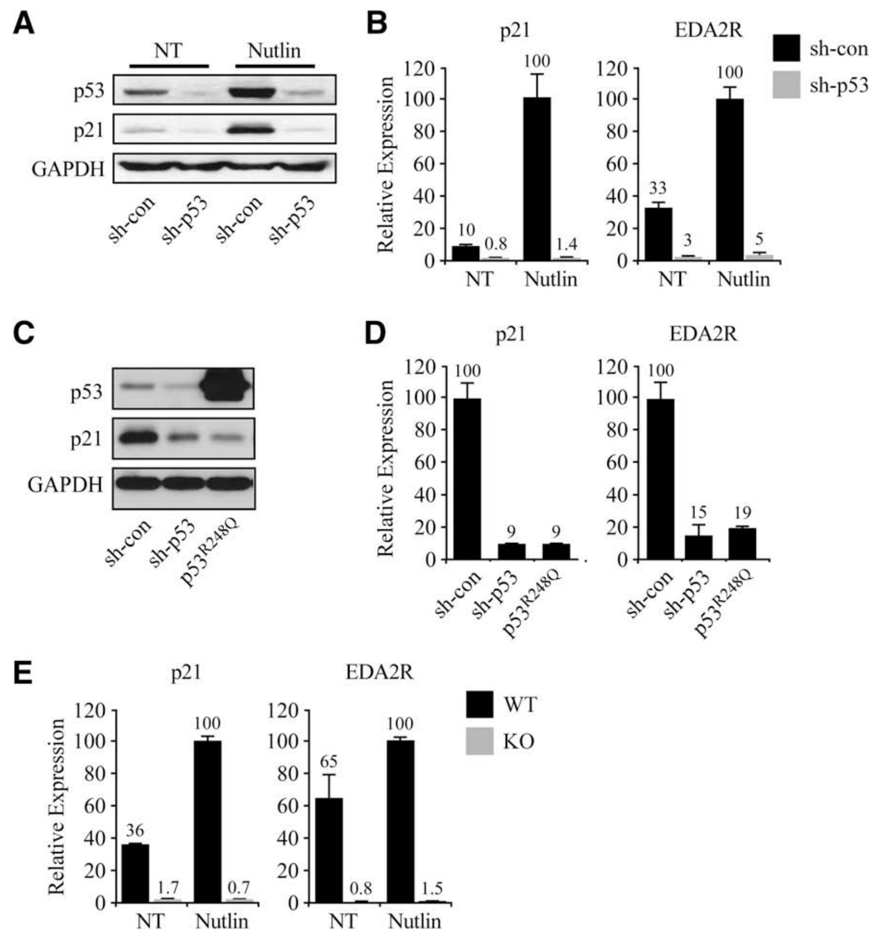

Fig. 1. *EDA2R* is transactivated by p53 in human fibroblasts. (A and B) sh-p53 and sh-con WI-38 cells were treated with Nutlin-3a (10 μM, 24 h). Protein levels were measured by Western blotting (A). mRNA levels of *p21* and *EDA2R* were measured by QRT-PCR (B). (C and D) sh-con, sh-p53 and mutant $p53^{R248Q}$-expressing IMR90 cells were assayed for p53 and p21 protein levels by Western blotting (C) and mRNA levels of *p21* and *EDA2R* by QRT-PCR (D). (E) Mouse embryonic fibroblasts, originating from either a WT-p53 or a p53-KO mouse, were treated with Nutlin-3a (10 μM, 24 h) and analyzed for *p21* and *EDA2R* expression by QRT-PCR.

death, as evident by reduced total cell amount and increased percentage of dead cells (Fig. 2). Similarly, EDA-A2 treatment of H1299 p53-null cells, which were first transfected with either WT-p53 or mutant p53, resulted in induction of apoptosis only in WT-p53-transfected cells (Supplementary Fig. 1E).

3.3. EDA2R is induced by p53 in chemotherapy-treated mouse skin and in human hair follicles

As mentioned above, the ectodysplasin family of receptors and ligands are implicated in the control of hair growth and in pathologies associated with abnormal hair growth. We therefore hypothesized that the induction of *EDA2R* by p53 might mediate the effect of p53 on hair growth and hair loss. Importantly, p53 was reported to be essential for CIA (hair-loss), a process involving p53-dependent apoptosis of hair follicles [17]. We therefore tested if *EDA2R* is induced by p53 during the process of CIA. To this end, we applied a specific experimental protocol [15] to chemically induce alopecia in WT-p53 and p53-KO mice. According to this protocol, the mouse dorsal fur is first depilated in order to synchronize hair growth. After nine days, when hair follicles reach the chemotherapy-sensitive stage, cyclophosphamide (CYP), a chemotherapeutic drug, is injected. Approximately one week later, the dorsal fur should be shed in CYP-treated mice. As shown in Fig. 3A, when WT-p53 and p53-KO female mice were treated according to this protocol, they displayed complete p53-dependent loss of their dorsal fur. i.e., p53-KO female mice did not shed their hair at all, while WT-p53 females displayed complete hair loss in the previously-depilated area. Surprisingly, in male mice treated under the same conditions, p53 had only a mild effect, slightly delaying the kinetics of hair loss. To the best of our knowledge, this is the first report of such clear gender-specific dependence on p53 in the context of CIA.

Importantly, *EDA2R* expression was significantly induced in a p53-dependent manner in the mice back skin approximately 2 days after cyclophosphamide injection (Fig. 3B). Interestingly, the expression of *p21*, which mediates cell-cycle arrest [18], was repressed. These results support the suggested role of *EDA2R* in mediating apoptosis of hair follicles. Of note, the p53-dependent induction of *EDA2R* in CYP-treated mice was not confined to the skin, and was also evident in the lungs (Fig. 3C) and liver (data not shown). Moreover, *EDA2R* was also transcriptionally activated by p53 following ionizing irradiation in several mouse tissues (data not shown).

Next, to test whether *EDA2R* is induced also in human hair follicles (HHFs) following chemotherapy, we analyzed samples of in vitro cultivated HHFs that were treated with the active derivative of cyclophosphamide, 4-hydro-peroxy-cyclophosphamide (4-HC) [19]. Analysis of mRNA extracted from ~20 pooled HHFs from two different individuals demonstrated that *EDA2R* and *p21* were upregulated following 4-HC treatment (Fig. 3D).

Finally, we tested whether EDA2R is required for CIA in mice. We obtained *EDA2R*-KO mice, which were previously generated

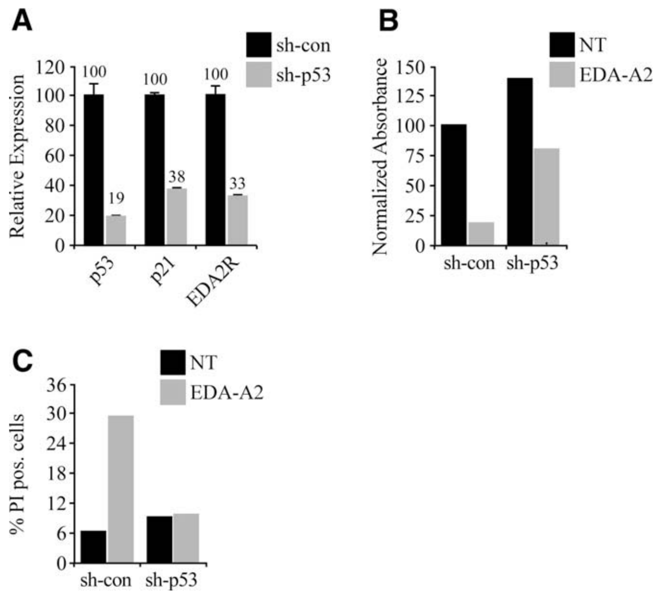

Fig. 2. EDA-A2 treatment results in p53-dependent cell death. (A–C) sh-con and sh-p53 U2OS cells were treated with EDA-A2. A. mRNA levels were measured by QRT-PCR in non-treated cells. (B) Estimation of cell amount by Crystal Violet. EDA-A2-treated and non-treated (NT) cells were stained with Crystal Violet and washed. Crystal Violet was then extracted with acetic acid and its concentration was quantified at OD 590 nm and the values were normalized to the sh-con non-treated sample. C. Analysis of cell death by Propidium Iodide exclusion. Cells were trypsinized and stained with Propidium Iodide. The percentage of positive cells, representing dead cells, was measured by flow cytometry.

be Newton et al., and were reported to be indistinguishable from their WT littermates [8]. We applied the same CIA protocol described above to test whether EDA2R-KO mice, similarly to p53-KO female mice, will display attenuated CIA. Interestingly, despite the p53-dependent induction of *EDA2R* in the dorsal skin of CYP-treated mice, our experiments did not demonstrate any significant attenuation of CIA, as EDA2R-KO mice displayed the same degree of hair-loss as their WT controls following cyclophosphamide administration (data not shown).

4. Discussion

The combination of previously published data and the results described above lead to the conclusion that *EDA2R* is a *bona fide* p53 target gene, which is activated in a wide variety of cell types and tissues of both human and mouse origin, and following a variety of p53-activating signals. Importantly, p53 transactivates *EDA2R* transcription in cancer cells and sensitizes them to EDA-A2-induced death, suggesting a therapeutic potential for this ligand. *EDA2R* is upregulated in vivo in a p53-dependent manner in the mouse skin, as well as in other tissues, following chemotherapy and radiotherapy. A similar phenomenon is apparent in human hair follicles. Since p53 activity is essential for CIA in female mice ([10] and Fig. 3), it is reasonable to assume that one or more p53 target genes are required for this process. Nevertheless, our results indicate that although *EDA2R* is induced by p53 during CIA, it is not necessary for the induction of hair loss, perhaps due to compensation by other pro-apoptotic p53 targets or even by p53-independent mechanisms. Supporting the first possibility; the p53 target gene *FAS*, which, similarly to *EDA2R,* encodes a member of the TNF-receptor superfamily; was reported to be activated during CIA, and its deletion decreases the levels of apoptotic markers in regressing hair follicles, but do not abrogate CIA [20]. Deletion of both *FAS* and *EDA2R* may potentially exert a greater attenuating effect on CIA than abrogation of each of them alone. Alternatively, it

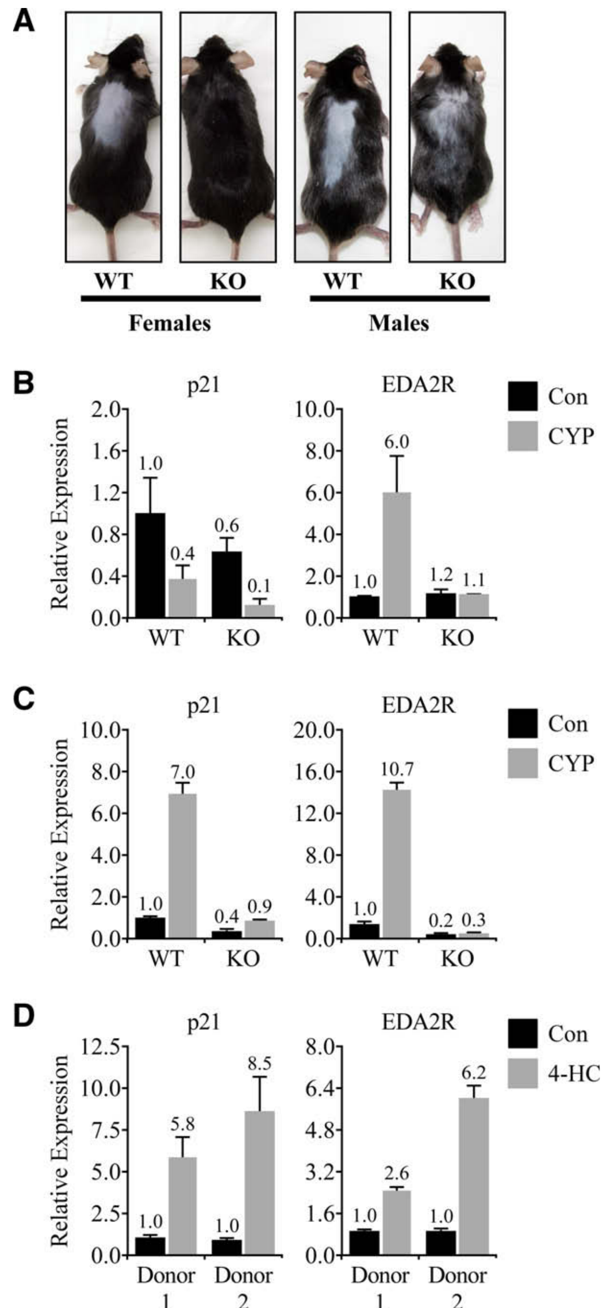

Fig. 3. EDA2R is upregulated following chemotherapy in a p53-dependent manner. (A–C) WT-p53 (WT) and p53-KO (KO) mice were depilated and treated with cyclophosphamide (150 mg/kg) 9 days later. (A) Representative images of treated male and female mice 8 days after cyclophosphamide treatment. (B and C) Expression of *p21* and *EDA2R* in dorsal skin (B) or lungs (C) 2 days after injection of Cyclophosphamide (CYP) or water (Con). (D) Expression of *p21* and *EDA2R* in in vitro cultivated human hair follicles derived from two different donors, 2 days after treatment with the active derivative of cyclophosphamide (4-HC) or control treatment (Con).

is also possible that p53 promotes apoptosis in hair follicles by trans-activating additional targets, or even by non-transcriptional activities [21]. Moreover, we demonstrate that in male mice, lack of p53 does not abrogate CIA, while in females, p53 is essential for this process. This clearly indicates a compensation for p53 loss in the context of CIA in males. In fact, some developmental defects associated with p53 deficiency, such as exencephaly, display low penetrance and appear most of the times in female embryos, indicating that in most cases, and particularly in males, p53 deficiency

is compensated, likely by p53 family members p63 and p73 [22]. Thus, it is possible that in the absence of *EDA2R*, p53 cannot initiate CIA, and this is compensated not by a different p53 target, but by a different pathway which is independent of p53. Finally, it is also possible that although *EDA2R* is elevated during CIA, this receptor does not initiate any signalling due to low concentration or absence of its ligand EDA-A2. Moreover, it was demonstrated in vitro that while high concentrations of EDA-A2 induce cell death [4], treatment with low concentrations can induce cell survival; and this induction is mediated by EDA2R-dependent activation of NF*k*B [23]. The antagonistic effects of EDA2R may thus result in a more complex scenario in vivo.

In sum, this study broadens our understanding of p53 function, and implicates p53 as a regulator of ectodysplasin signalling. Additionally, our data suggest a role for *EDA2R* in mediating p53-dependent cell death and offer a platform for further studies on the role of the p53 and EDA2R in CIA.

Acknowledgements

Supported by a Center of Excellence grant from the Flight Attendant Medical Research Institute. V.R. is the incumbent of the Norman and Helen Asher Professorial Chair Cancer Research at the Weizmann institute.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.058.

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